The Potential for Using Polygalacturonase (PG)-Inhibiting Proteins (PGIPs) as Part of a Strategy for Managing Pierce's Disease

Alan Bennett, Ann Powell, John Labavitch and others

The Potential for Using Polygalacturonase (PG)-Inhibiting Proteins (**PGIPs**) as Part of a Strategy for **Managing Pierce's Disease**

We based our work on PD Development on this model

Sharpshooter feeds

↓

***X. fastidiosa* presence**

↓

Host wall digestion, *Xf* systemic spread

↓

Oligosaccharide signals

↓

Host ethylene synthesis

↓

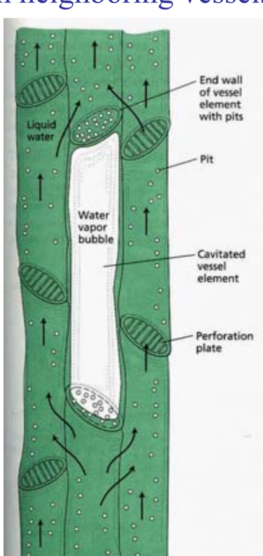
Xylem occlusion

↓

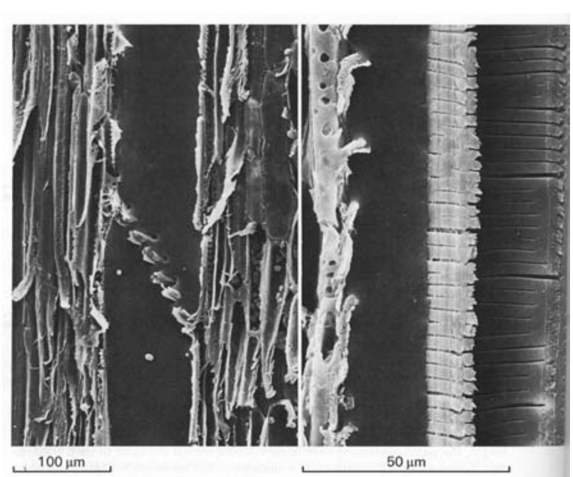
Collapse of water transport, leaf wilting and abscission, vine death

The key is in understanding how "systemic spread" of the *X. fastidiosa* population occurs.

Pit "membranes" are natural filters that partially separate one vessel from neighboring vessels or living parenchyma cells.



From: Taiz and Zeiger "Plant Physiology"



From: Zimmermann "Xylem Structure and the Ascent of Sap"

The *Xylella* genome contains:

➢ A single polygalacturonase (**PG**)-encoding gene and

➢ a few sequences that were putative endo-β-1,4-glucanase (**EGase**) enzymes.

Our PD model suggested that *Xf*'s plant wall-digesting enzymes contributed to the pathogen's systemic spread, **probably by degrading pit membrane barriers**.

UCD Plant Pathology Ph.D. student **Caroline Roper**:

➢ Cloned one EGase gene and the PG gene

➢ She expressed both the PG gene and the EGase gene in *E. coli*

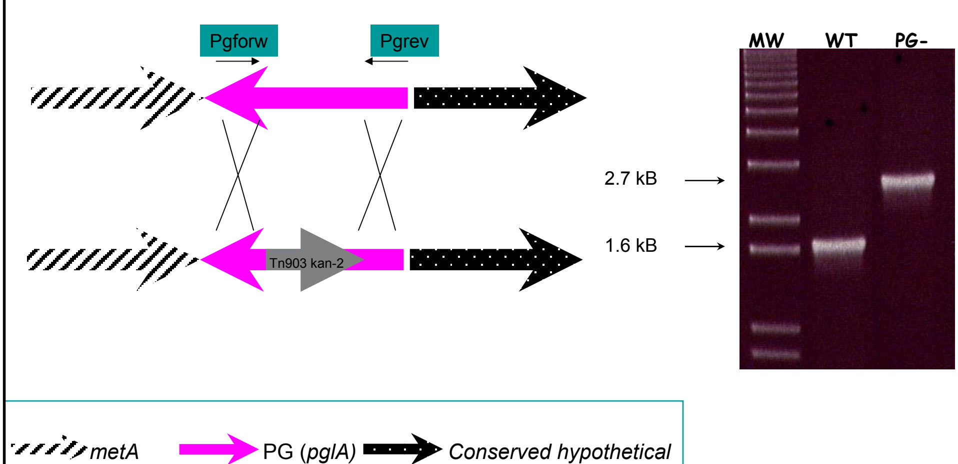
➢ Isolated protein from the *E. coli* and

➢ Demonstrated that the proteins had the predicted enzymatic activities. The **PG digested pectin**. The **EGase digested xyloglucan** as well as a cellulose substrate!

Are *Xylella*'s cell wall-modifying enzymes important for Pierce's disease development?

Caroline, working in Bruce Kirkpatrick's lab and mine, asked this question experimentally!

Caroline knocked out *Xf*'s PG gene, creating a **PG-less *Xf***.



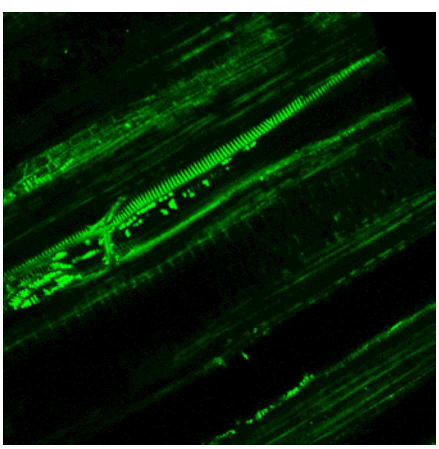
Pathogenicity Results

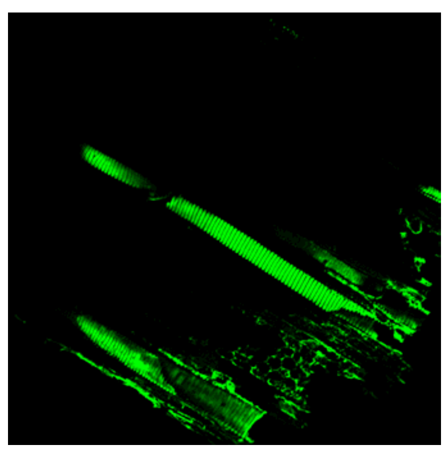
18 weeks post-inoculation

Wild Type *Xf* Fetzer — PG (-) *Xf* Fetzer — H_2O control

Without its PG, *X. fastidiosa* apparently does not cause PD!

If the bacterial population is to spread, we think that *Xf* must pass through the natural filters provided by "**pit membranes**".





Ca-bound pectin — **Xyloglucan**

Dr. Qiang Sun used **monoclonal antibodies** to establish the **identities of some of the grapevine pit membrane polysaccharides**.

In the previous slide you were shown immunological evidence that grapevine pit membranes contain:

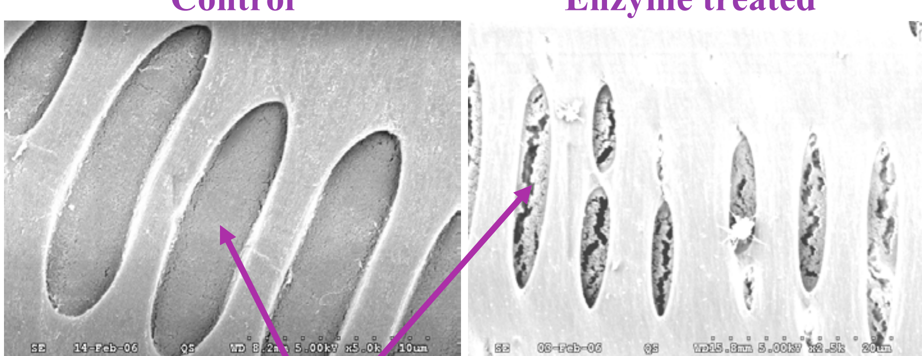
- Pectins (PG substrates) and
- Xyloglucans (substrates for β-1,4-glucanase)

Thus, one might imagine that the roles of *X. fastidiosa*'s PG and EGase are to break down the pit membranes and thus allow pathogen spread.

Vessel-Parenchyma Pits and Pit membranes

Following the introduction of PG and EGase to excised grapevine stem segments, Qiang Sun developed these images.

Control **Enzyme treated**



Pit membrane (face-on view)

MOLECULAR PLANT PATHOLOGY (2005) **6**(1), 43–51

Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene

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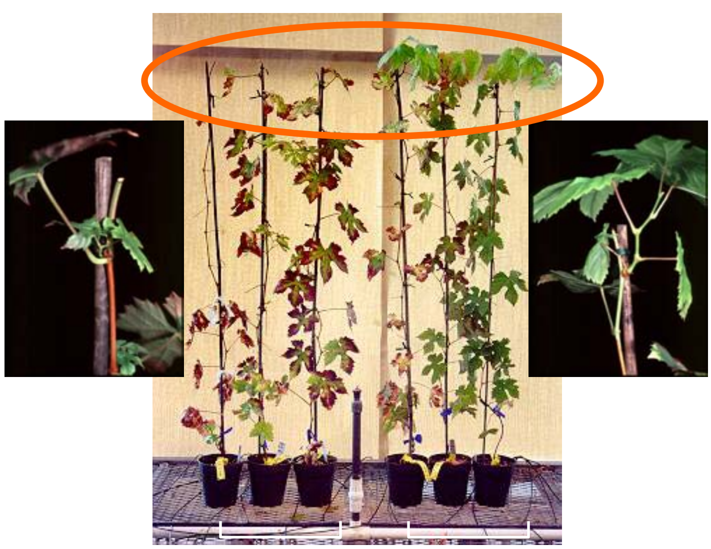
SUMMARY

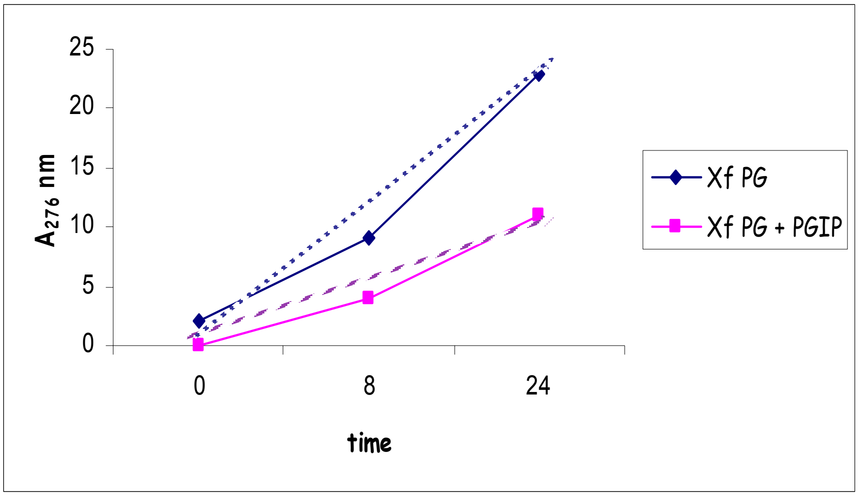
Polygalacturonase-inhibiting proteins (PGIPs) are plant cell-wall proteins that specifically inhibit fungal endo-polygalacturonases (PGs) that contribute to the aggressive decomposition of susceptible plant tissues. The inhibition of fungal PGs by PGIPs suggests that PGIPs have a role in plant tolerance to fungal infections and this has been observed in transgenic plants expressing PGIPs. *Xylella fastidiosa*, the causal agent of Pierce's disease (PD) in grapevines, has genes that encode cell-wall-degrading enzymes, including a putative PG. Therefore, we hypothesized that PGIP expression could confer tolerance against this bacterium as well as against the fungal pathogen *Botrytis cinerea*. To test this hypothesis, *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were transformed to express pear fruit PGIP-encoding gene (*pPGIP*) under the control of the CaMV 35S promoter. Substantial pear PGIP (pPGIP) activity was found in crude extracts from leaves and in xylem exudate of transgenic lines obtained from independent transformation events, but not in untransformed controls. pPGIP activity was detected in xylem exudate of untransformed scions grafted on to transgenic rootstocks expressing *pPGIP*. Leaves of transgenic plants infected with *B. cinerea* had reduced rates of lesion expansion. The development of PD was delayed in some transgenic lines with increased pPGIP activity. PD-tolerant transgenic lines had reduced leaf scorching, lower *Xylella* titres and better re-growth after pruning than the untransformed controls.

PGIPs are plant protein that selectively bind and inhibit pathogen PGs!

When PD develops on needle-inoculated control vines, symptoms are well developed in ca. 3 months. Symptom development on pear PGIP-expressing transgenics is substantially less.



Controls Transgenics



The cloned *Xf* PG was expressed in *E. coli* and the PG was tested for PG activity. Assay of PG (based on generation of reducing sugars when digesting pectin) shows that **the protein is a PG and is inhibited by pear PGIP**.

Transgenic expression of Polygalacturonase Inhibiting Proteins (PGIP)

Work from several different labs makes the RSAP optimistic that transgenic expression of PGIP could prove to be a very effective strategy for preventing PD. Because the *Xf* polygalacturonase gene has been cloned, and recombinant PG protein has been expressed, it is now possible to screen many different PGIPs for their ability to inhibitthe *Xf* PG enzyme. Although it has already been shown that pear PGIP is effective,there likely are PGIPs from other sources that are even more effective. Additional work remains to be done on optimizing delivery of PGIP to the xylem and on maximizing expression of PGIP in the cells surrounding the xylem. Greenhouse tests indicate that PGIP produced in the rootstock is translocated to the scion, but it remains to be shown that the levels of PGIP translocated are sufficient to confer protection against PD. Once this is established in the greenhouse, permits will need to be obtained to commence field trials.

RSAP review, pg. 18 & 19

As an alternative to transgenic expression of PGIP, it may be worth investigating the potential for small molecules to inhibit *Xf* PG. Would it be possible to develop an environmentally safe chemical that would effectively inhibit *Xf* PG activity and not grape PGs? Given that crystal structures are available for both bacterial PG's and plant PGIP's, there seems to be potential for rational design of such a compound. Ideally this compound would be taken up through roots, thus could be added to irrigation water. <u>The availability of recombinant *Xf* PG protein should make it feasible to develop a high throughput screen for testing readily available chemical libraries.</u>

Because of the apparent importance of the *Xf* PG, two strategies for potential PD control were recommended for further study: Inhibition of the bacterial PG by PGIPs or by other proteins or small molecules. Both approaches start with **production of PG inhibitors in transgenic rootstocks**.

Success in this project will entail contributions from several individuals and laboratories

- **Identification of technologies that are most likely to bring improved rootstocks into vineyards**
- **Optimization of PGIP production in and export from root tissues**
- **Production of sufficient *X. fastidiosa* PG to support extensive screening of molecules that may inhibit the PG**
- **Identification of (1) the PGIPs that are most effective at inhibiting *X. fastidiosa*'s PG and, then, (2) the features of those PGIPs that make them effective**
- **Generation of transgenic rootstocks and testing their ability to suppress PD development following infection**

If transgenic plant material is to be involved, important considerations include:

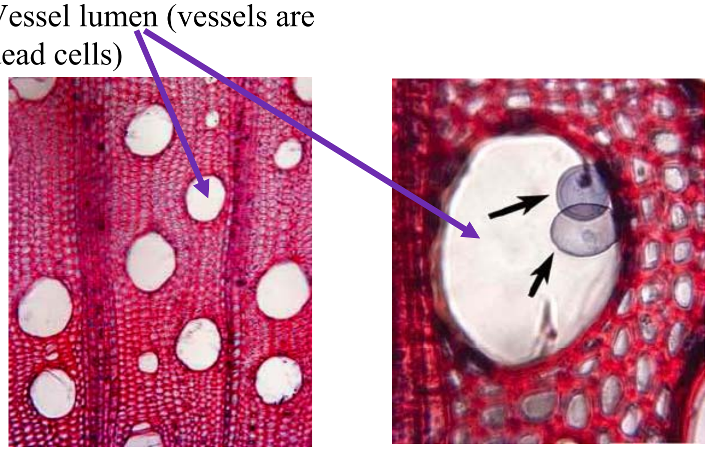
- **Will wines from scions grown on transgenic rootstocks be acceptable to consumers?**
- **Can those wines be called 'Chardonnay", Merlot etc.?**
- **Are the technologies that will be required to generate the improved rootstocks "accessible" to the industry?**

Our project will address the third question. The "industry" and the public must find answers to the others

Q: Are the technologies that will be required to generate the improved rootstocks accessible to the industry?

A: PIPRA, led by Alan Bennett and colleagues, is already supported to survey the "IP Landscape" related to other PD research projects. Issues related to ownership of PGIPs, technologies for transformation of grapes, promoters, signal sequences etc. will be examined. Ultimately an implementation strategy for a "PGIP-improved" rootstock will utilize technologies already included in PIPRA's portfolio, thus facilitating licensing and use.

Vessel lumen (vessels are dead cells)



Live parenchyma cells surround the vessel and "communicate" with the vessel system. Shown on the right are tyloses ballooning into the vessel lumen from the parenchyma cells.

These micrographs are from Sun, Rost, Reid & Matthews (Plant Physiology, in press)

Living cells in root tissues will express PGIP genes.

Q: How do we assure that PGIPs are produced at a high level?

A: Doug Cook and David Gilchrist (UC Davis, Plant Pathology) have promoters that are highly expressed in xylem parenchyma cells. **Key questions are:**

- **Are they expressed in grapevine root xylem parenchyma?**
- **Do they work when linked to PGIP genes (the PGIPs that are the best inhibitors of *X. fastidiosa* PG, in particular)?**

Living cells in root tissues will express PGIP genes.

Q: How do we assure that PGIP made in the xylem parenchyma cells is loaded into the vessels and moved to the scion?

A: Aguero et al. (2005) found that pear PGIP made in transgenic grapevines was exported to the xylem and was passed from transgenic roots into non-transgenic scions!

Q: Can this be improved on?

A: We think so. On-going and proposed work from Dandekar, Gilchrist et al. is aimed at identifying and then testing the utility of signal sequences that can enhance transport of proteins into the apoplast. For xylem parenchyma cells, a big part of the apoplast is the vessel lumen!

The RSAP Review stated “The availability of recombinant *Xf* PG protein should make it feasible to develop a high throughput screen for testing… “

The current system to provide *Xf* PG for testing involves expression of the PG gene in *E. coli*. It must be improved.

Q: How will improvements be made?

A: In a continuing project, Kirkpatrick et al. will be modifying the *E. coli* system to avoid the problems with PG precipitation & activity loss. In the new project we will collaborate with a researcher adept in the use of many systems for expression of recombinant proteins.

Q: How will “optimized” PGIPs be identified so that the best selections are tested in transgenic rootstocks”?

A: Ann Powell (UCD) a PI on the new project, has already been expressing PGIP genes from several plant species in *A. thaliana* The PGIP proteins will be collected and tested for their ability to inhibit *Xf* PG. Additional sequences will be identified and expressed. Prof. Melane Vivier (Inst. For Wine Biotech., Stellenbosch U.), a colleague of ours, has worked with PGIPs from *V. vinifera* and other grapes for many years. These PGIPs may be effective against the *Xf* PG. Melane and her colleagues will be part of our team.

Will this screen give the "best" PGIP for the job?

Biochemistry **2002,** *41,* 10225−10233

Use of Amide Exchange Mass Spectrometry To Study Conformational Changes within the Endopolygalacturonase II−Homogalacturonan−Polygalacturonase Inhibiting Protein System[†]

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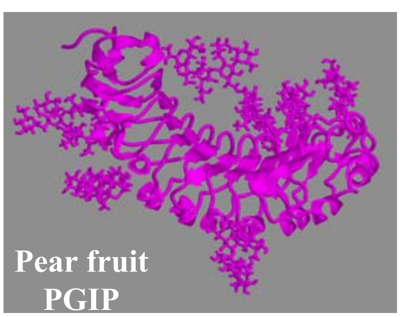
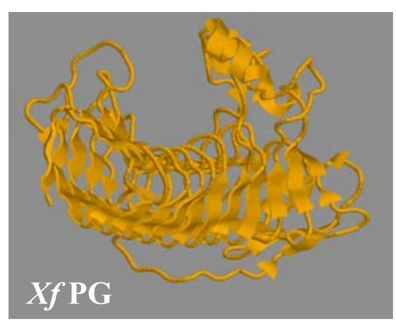
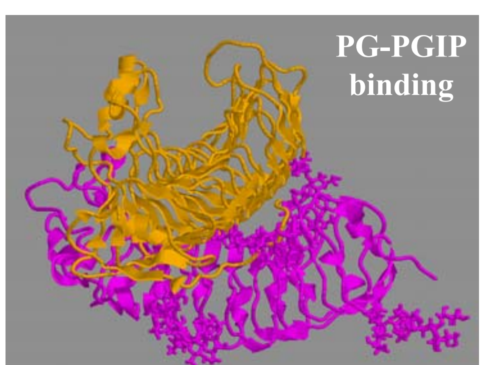
ABSTRACT: Amide exchange mass spectrometry (MS) was used to study the enzyme endopolygalacturonase II (EPG-II) from *Aspergillus niger* as it binds to an oligosaccharide substrate. A localized decrease in the level of deuterium incorporation in EPG-II of the EPG-II−oligosaccharide complex relative to that of the free EPG-II identified the location of substrate contact, which is in agreement with published site specific mutation studies. In addition, when bound with substrate, regions of EPG-II remote from the substrate binding site became exposed to the solvent, as revealed by an increase in the amount of incorporated deuterium, indicating a conformational change in the enzyme. Fluorescence experiments were performed to provide additional evidence for an altered conformation of EPG-II as a result of substrate binding. This novel application of amide exchange-MS to the study of protein−carbohydrate binding has, for the first time, described in detail the conformational changes associated with EPG-II when it binds a substrate. Amide exchange-MS was also used to study the interactions of EPG-II and the polygalacturonase inhibitor protein (PGIP). Mass spectral data of the EPG-II−oligosaccharide complex in the presence of *Phaseolus vulgaris* PGIP indicate that the inhibitor contacts EPG-II at a site remote from the substrate binding cleft, and is restricting the conformational changes of EPG-II. Fluorescence experiments also revealed that upon binding of PGIP, the conformational changes mentioned above for the EPG-II−substrate complex are minimized. These results, together with previously reported data, point to a location on EPG-II for interaction with PGIP as well as a possible mechanism for noncompetitive inhibition of EPG-II.







Images from Dan King

Tests will **identify different PGIPs that are either good or bad at inhibiting the *Xf* PG PG**.

Then, **modeling of the *X. fastidiosa* PG structure and its interactions with PGIPs will identify details of PGIP structure that are important for effective inhibition**.

In the longer term this could lead to the **engineering of a PGIP protein optimal for grapevine PD protection**.

The modeling would also **inform work aimed at identifying small molecules that can inhibit *Xf*'s PG** (Kirkpatrick).

Transformation of rootstocks to express selected PGIPs will utilize available **PIPRA technologies** to minimize "IP encumbrances". Transformation constructs will include **combinations of different rootstock germplasm, PGIP genes, promoters and apoplast targeting sequences**.

- **Subsequent testing will examine**:
- **Rootstock growth and scion performance**
- **PGIP gene expression and protein levels**
- **PGIP delivery to scions**
- **Ability of the rootstock-expressed PGIP collected from scion xylem fluid to inhibit *Xf* PG**

And the **best performers** (i.e., **paired scions and transgenic rootstocks**) will be tested for:

- **Decreased PD susceptibility**, and then
- **Fruit development, growth, grape and wine quality**, etc.